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Understanding the molecular basis of breast carcinoma is central to designing rational and effective therapy for this prevalent disease. We have proposed to investigate several regulatory proteins that we hypothesized may underlie at least a subset of human breast carcinomas. In investigating transcription factors of the POU domain class and their synergistic interactions with nuclear receptors, we have discovered a novel protein, expressed in the breast, that acts as a specific repressor of retinoic acid and thyroid hormone receptors. This protein is a 270kDa protein that is postulated to mediate retinoic acid and thyroid hormone receptor actions as a repressor of specific gene expression. The retinoic acid receptor is known to exert critical actions in controlling cell proliferation, making it an ideal candidate for initiating events in breast tumors. This co-repressor protein, normally serving to control mammalian cell proliferation, and the maintenance of mature cell phenotypes, represents an intriguing candidate for involvement in initiation of tumor events.

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Introduction

Breast cancer is a devastating disease, the etiology of which may be best understood by investigating the regulatory events that can maintain or restore normal growth properties. Our laboratory has investigated control of gene transcription and mammalian development, emphasizing two classes of transcription factors: POU domain factors, and nuclear receptors that mediate both positive and negative patterns of gene expression. Our ongoing studies under this Award are based on the premise that these factors, and trophic receptors under their control, are of specific significance to the etiology of breast cancer. While nuclear receptors clearly are important in the biology of breast tumors, the retinoic acid receptor appears to exert critical roles in normally preventing abnormal proliferation events. Altered control by defects in the regulation of the retinoic acid receptor is likely to serve in initiation events in breast cancer. In turn, retinoic acid receptor appears to require a co-expression to mediate its normal control of growth. Under this grant, we have identified a novel protein that appears to be the long-sought retinoic acid receptor co-repressor (RRCR), the function of which is regulated by allosteric effects imparted by the DNA sites to which retinoic acid receptor is bound. Thus, on specific DNA sites, this protein associates with, and is released by binding of ligand, while on other sites it is not released by binding of ligand. This novel molecule has clear implications with respect to initiation of abnormal growth and loss of differentiation in mammary epithelium. Based on the nature of the specific DNA sites to which retinoic acid receptor is bound, this factor is postulated to be a central aspect of the ability of nuclear receptors to promote growth in some cell types, and inhibition of growth in others, we hypothesize that environmental conditions and genetic predisposition alter regulation of this factor.

The morphogen retinoic acid is required for development, growth and differentiation (reviewed in 1 and 2). *Retinoids, a group of analogs of vitamin A, particularly at high levels, suppress carcinogenesis in various epithelial tissues, including the mammary gland* (3-6). We believe that this reflects the actions with a co-repressor that we believe may serve roles in initiation of breast cancer. The hormone effects are mediated by binding to specific nuclear receptors (3) that are members of the steroid/thyroid hormone receptor superfamily (4-6). This class of proteins function as ligand dependent transcription factors that mediate the response of the hormone signal by direct control of gene expression. Unlike the estrogen and glucocorticoid receptors that bind DNA as homodimers, retinoic acid receptors preferably interact with their cognate DNA response elements as components of heterodimeric complexes (7-20) often involving a partner that our laboratory and others identified to be members of the retinoid X receptor (RXR) family (8-21). Heterodimers of retinoic acid receptor and retinoid X receptor bind with high affinity and activate transcription from response elements consisting of direct repeats, palindromic, or inverted palindromic arrangements of a core recognition motif that has the consensus sequence NNAAGGTCA (4, 8-21). The relative orientation and spacing of the core recognition motifs play essential roles in the specificity of DNA binding and transcriptional activation. While heterodimers of retinoic acid receptor and retinoid X receptor bind to direct repeats of core motifs spaced by 1, 2 and 5 bp (DR+1, DR+2, and DR+5), an unspaced palindrome binds to an inverted palindromic arrangement of the core motif spaced by 6-8 bp, (e.g. IP+6) (reviewed in 22, 23).

Recent studies indicate that heterodimeric complexes of retinoic acid receptor and retinoid X receptor molecules exhibit a polarity binding to various DNA elements (24-26). This polarity-specific binding may play important roles in cell-specific regulation by retinoic acid receptors. On a direct repeat spaced by 5 bp (DR+5) retinoid X receptor selectively binds to the upstream half-site and retinoic acid receptor binds to the downstream half-site (25,26). When the spacing is reduced by one basepair (DR+4 site) the element becomes a binding site for heterodimers of thyroid hormone receptor and retinoid X receptor. In this case the thyroid hormone receptor is bound to the downstream half-site and the retinoid X receptor again interacts with the upstream half-site. However, in the case of a direct repeat spaced by 1 bp (DR+1) retinoid X receptor binds to the downstream half-site and retinoic acid receptor binds to the upstream half-site (27). The stringency of this polarity-specific binding was further confirmed using specific mutants of retinoid X receptor containing the P-box residues of the glucocorticoid receptor (27).

In contrast to the glucocorticoid receptor, retinoic acid receptors are not associated with heat shock proteins in the absence of hormone, but are bound to their response elements and are able to actively repress basic transcription (28,29). Our laboratory and others, were able to show that this repression is mediated by the C-termini of the retinoic acid and thyroid hormone receptors (30-32).

Thyroid hormone and retinoic acid receptors belong to the nuclear receptor family of ligand-regulated transcription factors and serve to modulate specific developmental and homeostatic programs of gene expression, by binding to cis-acting DNA sequences in target genes (33). These receptors bind as heterodimers with the retinoid X receptor (36-40,44) in a polarity-specific fashion to cognate DNA sites that are generally organized as direct repeats of a core binding motif (17,41-43). As a consequence of binding to these sites, thyroid hormone and retinoid acid receptors can exert both positive and negative control of gene transcription.

The thyroid hormone receptor was originally identified based on homology to the viral oncogenes erbA of the Avain Erythroblastosis Virus (AEV). V-erbA, which in contrast to the wild-type receptor does not bind thyroid hormone with high affinity, was demonstrated to function as a constitutive transcriptional repressor of both thyroid hormone and retinoic acid responsive genes (34,35,45). The cellular thyroid hormone receptor was also found to repress transcription of target genes in the absence of ligand, with hormone binding resulting in de-repression and activation (31,34,35). While in some cases, ligand-independent repression could involve simple competition for common or overlapping DNA binding sites, or involve competition for a common heterodimeric partner, several lines of evidence indicate that in most cases ligand-independent repression appears to result from an active repressor functions within the ligand binding domain. A ligand-independent repression function could be transferred by the carboxyl-terminal region of the thyroid hormone receptor to heterologous DNA binding domain. Fusion of the C-terminal domains of v-erbA, T3R, and RAR to the DNA binding domain of the yeast transcription factor GAL4, generated UAS-dependent transcriptional repressor proteins (31). In contrast, the RXR C-terminus fused to the GAL4 DNA binding domain did not mediate transcriptional silencing.

The molecular mechanism responsible for nuclear receptor transcriptional silencing are not well understood. Recent studies showed that several nuclear receptors may interact with the basal transcription factors, including TFIIβ (47). The thyroid hormone receptor interacts with TFIIβ through two distinct regions. The N-terminus of T3Rβ interacts in a ligand-independent fashion with the repeat motif of TFIIβ, while the C-terminal portion of the ligand binding domain interacts with the zinc finger motif at the N-terminus in a manner that it is released by ligand. Unliganded thyroid hormone receptor inhibits nuclear transcription *in vitro* by preventing formation of the preinitiation complex. Further data however, indicate that the distal T3 receptor thyroid hormone C-terminal regions that interact with TFIIβ, are not sufficient to confer repression. Indeed, the regions in the hinge and N-terminal part of the ligand-binding domain of the thyroid hormone receptor are required for silencing (46,47). Co-transfection experiments suggest that these sequences which do not bind TFIIβ can potentially compete for a putative soluble co-repressor molecule (47) and imply the existence of additional interacting factors that are required for ligand-independent repression.

Over this year, we have identified a single 270 kDa protein that associates with DNA-bound thyroid hormone, retinoid X receptor heterodimers, in the absence of thyroid hormone, but not in its presence. Isolation of the cDNA encoding this factor has revealed a novel nuclear repressor, characterized by an interaction domain in the distal C-terminus, and a transferable repressor domain in the N-terminus. This factor interacts with the thyroid hormone receptor is the mutant cell and also interacts with retinoid acid receptor, but not with the estrogen, retinoid X, glucocorticoid, or vitamin D receptors. Receptor specificity is explained by a conserved region located in the hinge region between the DNA-binding and ligand-binding domains that is present in all thyroid hormone and retinoic acid receptors as well as in v-erbA. Specific mutations in this region that abolished interactions with the 270 kDa protein also eliminated the ligand-independent repression function of the thyroid hormone receptor. The 270 kDa protein itself function as a repressor. Together these data suggest that the 270 kDa protein associated with the unliganded, DNA-bound thyroid hormone receptor, and retinoic acid, is required for ligand-independent transcriptional repression; we have therefore termed this protein N-CoR for nuclear receptor co-repressor.

Body

As has been previously described, the C-terminus thyroid hormone receptor β1 (T3R-β1) receptor transferred active repression when fused to the Gal4 DNA binding domain. Thus, the critical information required for repression resides in the C-terminus of the various forms of the thyroid hormone receptor. To

begin to investigate the mechanism by which the thyroid hormone receptor can repress gene expression, we evaluated the ability of specific factor(s) to associate with the thyroid hormone receptor when bound, as a heterodimer, to its cognate DNA site. A biotinylated thyroid hormone receptor DNA response element was utilized to assemble stable thyroid hormone/retinoic X receptor heterodomain on a streptavidin agarose matrix. Whole cell extracts prepared from CV-1 cells were then added and incubated at $4^{o}C$; following extensive washing, bound material was eluted, fractionated by SDS polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Thyroid hormone receptor-associated protein were identified using ^{32}P-labeled thyroid hormone receptor C-terminus prepared from bacteria. This binding assay revealed that a single 270 kDa protein (p270) was capable of selectively binding to the unliganded thyroid hormone receptor. Addition of ligand released p270 (Brown/Parker); conversely, the previously identified 140 kDa and 160 kDa proteins were now associated with the thyroid hormone receptor heterodimer.

Isolation of the cDNA encoding the p270 factor

In order to isolate the cDNA encoding the p270, we utilized the yeast two-hybrid screen. The DNA sequences corresponding to the carboxyl-terminal domain of the rat thyroid hormone receptor alpha (amino acids 122-410) were cloned into the yeast two-hybrid bait vector, in frame with the LexA DNA binding domain. The yeast two-hybrid prey vector containing an oligo-dT-primed HeLa cell-derived cDNA library was co-transformed with the bait vector into yeast and plated onto galactose/X-gal-agar plates with appropriate auxotroph selection. One hundred positive blue colonies were picked and tested for interaction in the absence and presence of the thyroid hormone receptor ligand Triac. Seven clones interacted only in the absence of Triac and were further evaluated for interaction strength and specificity. Liquid cultures were prepared and harvested and β-galactosidase units were measured. One clone interacted considerably more strongly than the other six (1500 β-gal units vs<1000 β-gal units). Using the cDNA recovered from their clone, both HeLa and mouse pituitary cDNA libraries were screened, and rescreened, until the entire coding sequence was obtained and sequenced. The murine cDNA contained a 7359 nucleotide open reading frame that predicted a 2453 amino acid protein. An alternatively spliced variant, which results in a loss of a 2333-2371, was also detected. No significant homologies with any other proteins in the available data bases were detected, but a potential Cys, Cys-His, His zinc finger motif was noted at aa 965-980.

The specificity of interaction with the T_3 receptor C-terminus was evaluated by examining interactions with other nuclear receptors, both in yeast and *in vitro*. In addition to strong interactions, as recorded by the yeast two hybrid assay with the T_3 receptor C-terminus, the factor also interacted with the comparable region of the retinoic acid receptor α(RAR). In both cases, interactions were abolished upon the addition of ligand. In contrast, no evidence of interactions were detected with the C-termini of the estrogen, glucocorticoid, vitamin D, or retinoid X receptors (RXR). Direct binding was assessed by GST "pull-down" assays, utilizing that $T_3R\beta1$, and RARα1, and RXRα fusion proteins, using either the 724 aa C-terminal region corresponding to aa 1829-2453, or the 270 kDa holoprotein expressed by *in vitro* transcription and translation in reticulocyte lysate. Strong interactions were noted with both the thyroid hormone and retinoic acid receptor C-termini, in agreement with the yeast two hybrid assays, while there was no or minimal interactions with the retinoid X receptor C-terminus. Although addition of Triac (3,3',5-triiodothyroacetic acid) produced a significant decrease in binding, addition of retinoic acid did not result in significant reduction of binding to the retinoic acid receptor in selection. Intriguingly, T_3 also failed to release the 270 kDa protein. In contrast, on DNA, both T_3 and retinoic acid from GST-TR of the 270 kDa protein. These data suggest that the configuration of the heterodimer on DNA is a critical aspect of the ligand-dependent release of the 270 kDa protein and that the 270 kDa protein is apparently bound to the thyroid hormone receptor, but not retinoid X receptor of the DNA associated heterodimers.

We next investigated whether the cloned 270 kDa factor was a nuclear protein associated with thyroid hormone receptor in the cell and whether it corresponded to the p270 associated with DNA-bound thyroid hormone receptor. We constructed a transcription unit expressing the cloned 270 kDa protein with a C-terminal in-frame FLAG epitope, and generated a guinea pig antisirum against aa 811 - 996 of the cloned protein. Whole cell extracts were prepared from COS cells expressing the p270-FLAG protein and monoclonal antibodies against the N-terminus of thyroid hormone receptor β1 were utilized to immunoprecipitate T3-receptor and any

associated proteins. The 270 kDa factor was specifically immunoprecipitated in a T3 receptor-dependent fashion; therefore, the interaction between the 270 kDa protein and thyroid hormone receptor occurs in the cell. To determine whether p270 binds to both TR and RAR protein interactions assays were performed with RAR-RXR heterodimers bound to DNA. The 270 kDa protein pulled down in a TR-dependent fusion was detected by 32p-RAR, indicating that the identical protein interacts with both receptors. In order to further confirm that the factor, encoded by the cloned cDNA corresponded to the p270, bacterially-expressed GST-RAR or TR fusion proteins were utilized to purity associated proteins from L cell nuclear extract. These experiments revealed that the p270 protein was detected of anti N-CoR. Together, these data indicate that the cDNA clone encodes the p270 protein. Transfection of COS cells with the vector expressing the 270 kDa protein with the antigenic C-terminal FLAG epitope tag permitted histochemical determination that this protein was localized in the nucleus. Thus, the critical criteria for the functional interactions between nuclear receptors and p270 kDa protein have been fulfilled.

The gene encoding the 270 kDa factor appears to be widely expressed but at significantly different levels in various tissues and cell lines; RNA blot and RNase protection assays revealed expression in cell lines of diverse embryonic origin and in stem cells. While no line is entirely deficient in this transcript, cells expressing very low levels of functional protein have been identified.

p270 Interacts with the Hinge Region of the Thyroid Hormone Receptor

Using a series of N- and C-terminally truncated proteins, the interaction domain in the 270 kDa protein was localized to a 60 amino acid region in the C-terminus. A further N-terminal deletion in the human homologue suggests that the critical region is encompassed by a 45 amino acid sequence (2255 to 2300). The algorithms described by Chou-Fassman and Garnier-Osguthorpe-Robson predict an α-helical region in the C-terminus of this interaction domain.

In order to identify the sequences in the thyroid hormone receptor that were required for interaction with p270, we expressed the C-terminal 397 amino acids of the protein fused to GST and tested for interaction with N- and C-terminal deletion mutants of T3R-β1. C-terminal deletions to amino acid 335 had only a limited effect on binding, while deleting beyond aa 260 completely abolished the interaction. Internal deletion of between amino acids 260 and 335 sequences also maintained binding, suggesting that the hinge and N-terminal portion of the ligand binding domain mediates binding, while the surrounding sequences might modulate the availability of this domain for interaction. This hypothesis was tested using a series of GST-T3Rβ fusion proteins containing sequential N-terminal truncations. Each bacterially expressed protein was tested for binding of 35-labeled protein encompassing the C-terminal 724 amino acids of N-CoR. Interactions were maintained with successive deletion of the N-terminus, DNA binding domain, and the T/A-region . While information N-terminal of residue 203 was not required for interaction, deletion to amino acid 230 abolished binding. These observations revealed that the critical binding region involves the hinge region (aa 203-230), probably with additional contributions from the initial portion of the ligand binding domain (aa 230-260). Comparison of the sequence of the thyroid hormone and retinoid acid receptors, which interact with the 270 kDa protein, and of other nuclear receptors that do not interact, revealed a region that shows a significant homology (8 of 17 residues entirely conserved) only between the two nuclear receptors that showed ligand-independent interaction with N-CoR, v-erbA and the vitamin D receptor. Based on the homology, a series of mutations were generated in the CoR box and interactions with p270 were evaluated in the context of the full T3Rβ1 holoprotein, or the full receptor C-terminus. Mutants were usually involved substituting conserved residues both amino acids that might alter the helical properties of this region. Mutant proteins were initially evaluated for maintaining normal heterodimeric DNA binding properties. Mutations of E215->R and A,H,T 223,224,227->GGA were selected for more extensive analysis based on the observation that DNA binding was comparable to the wild-type receptor. These were evaluated for their ability to bind the C-terminal 725 amino acids of N-CoR. N-CoR actively bacterially-expressed by GST T3-receptor C-terminus. The E215->R bound to the 270 kDa factor equivalent to the wild-type receptor. However the A,H,T 223,224,227->GGA T3-receptor was very ineffective in binding the 270 kDa protein. These data were confirmed by similar assays using ^{35}S-labeled holo N-CoR.

Based on these results, the wild type, E215->R, and A,H,T 223,224,227->GGA thyroid hormone receptors were utilized to evaluate whether a receptor variant that was incapable of binding the 270 kDa protein was selectively impaired in its capacity to mediate ligand-independent repression. The E215->R mutant exhibits full capacity to inhibit transcription, while the A,H,T 223,224,227->GGA receptor was incapable of exerting repression function. Protein expression, determined using T3-receptor antibody was equivalent for the wild type and variant receptors. To confirm the specificity, variant and wild-type receptors were evaluated for their ability to function as activator; indeed, T3-dependent activation was actually somewhat higher with the A,H,T 223,224,227->GGA mutant T3-receptor than with the wild-type receptor.

Based on these observations, we postulate that the 270 kDa protein functions as the co-repressor of the thyroid hormone and retinoic acid receptors, because it is the only protein binding to DNA-associated T3 receptor in the absence of ligand, and receptor mutations that eliminate the binding to the 270 kDa protein simultaneously abolish ligand-independent transcriptional repression. We therefore refer to this protein as the nuclear receptor co-repressor, N-CoR and the region to which it bind in the T3 receptor the CoR-box.

If this model were correct, N-CoR would be predicted to be capable of conferring repression if fused to a heterologous DNA binding domain. To perform this experiment, N-CoR was fused to the GAL4 DNA binding domain, and the ability to serve as repressor was evaluated in several promoter contexts. Those experiments revealed that N-CoR, on binding DNA, mediates a 7-to 15 fold repression, depending on promoter context and cell type. In order to test this prediction, a series of 10 fragments of 300-400 aa, spanning the entire protein, were used to generate GAL4-fusion proteins. Transcription units expressing each protein were utilized for co-transfection assays with the luciferase reporter under the control of the TK promoter and three 5' GAL4 binding elements. The extreme N-terminal fragment consistently transferred a 7-to-15 fold repression in every assay and in all cell types evaluated. A smaller repression function could be transferred by a second region (residues 752-1116), which encompasses a potential zinc finger motif. None of the fragments transferred significant activation of transcription. The N-terminal region contains both glutamine rich regions and several regions rich in basic amino acid residues, both implicated in repressor domains of other proteins. Indeed, we find the initial 150 amino acids of this region transfer (~5-fold) the repression activity.

Conclusions

In addition to their well-established roles in ligand-dependent activation, nuclear receptors can serve functional roles as active repressors. Transcriptional repression by thyroid hormone and retinoic acid receptors occur in absence of ligand, when they are bound as heterodimers with retinoic X receptor to their cognate DNA sites. Previous studies have suggested the existence of a co-repressor required for ligand-independent silencing functions of the thyroid hormone and retinoic acid receptors, our data indicate that the co-repressor is a 270 kDa protein that selectively interacts with DNA-bound T3R/RXR heterodimers and which is released on binding of thyroid hormone. This 270 kDa protein is also associated with the retinoic acid receptor, for which ligand-independent repression has been described, but not with a series of other nuclear receptors for which such repressive events have not been reported.

The 270 kDa protein specifically interacts with a region in the hinge between the DNA and the ligand binding domain that is well-conserved between all thyroid hormone and retinoic acid receptors, but not other receptors. This region is adjacent to the T/A-region that exerts critical functions in DNA site-selective binding. This region, which we refer to as CoR-box, identifies an unsuspected regulatory domain in what was previously considered to be a poorly conserved region in the C-terminus of nuclear receptors. Indeed, by Chou-Fasman and Garnier-Osguthorpe-Robson algorythms, this region is suggested to form an amphipatic helix region, and it is likely that coiled, coil interactions with a helical region within the 45 aa interaction domain of N-CoR are required for the high affinity protein interactions within the cell. Because N-CoR fails to interact with vitamin D receptor which is exhibits high conservation within the CoR box, there are probably additional requirements for high affinity interactions with the N-terminal portion of the ligand binding domain, and the ability to effectively bind the 270 kDa protein is effectively regulated by ligand binding on DNA-bound heterodimers. These observations are consistent with the findings of Baniahmad et al., who demonstrated that this region of the

thyroid hormone receptor overcomes repression *in trans*, and suggested that it interacted with a critical co-repressor. Mutations of specific residues in the hinge helical domain that prevent binding of the 270 kDa protein also selectively prevent ligand-independent repression. However, ligand-dependent activation function remains intact. Independent confirmation of the critical role of p270/N-CoR is suggested by studies of the retinoic acid receptor, in which N-CoR has been demonstrated to control DNA site-specific responses to activating ligand. The inability of RAR to activate transcription when bound to a DNA site, on which it exhibits an opposite polarity of binding compared to the DR5 site, is based on the apparent inability of RAR specific ligand to disunite N-CoR from RAR on this site. These observations demonstrate that the interaction of N-CoR with RAR is also regulated by the DNA response element, and reveals that repressor function is dominant to ligand-dependent activation events. Because alternative nuclear receptors such as LXR, and NGFIB can heterodimerise with RXR on identical sites, only a subset of nuclear receptors binding to specific DNA sites will mediate ligand-independent repression. The function of v-erbA as a repressor exemplifies the potential roles of this repressor function in control of abnormal proliferative events, as well as in normal homeostasis. Indeed, $T_3R\alpha 2$ is highly expressed in specific tissues, while lacking the C-terminal sequences required for ligand-binding or activation, this receptor contains all the information required for DNA binding and N-CoR function. This implies that N-CoR serves critical developmental and homeostatic functions, ligand-dependent which is the case of $T_3R\alpha 2$ will not be relieved by ligand, consistent with the documented function of $T_3R\alpha 2$ receptor as a constitutive repressor.

The characterization of the N-CoR protein, based on the cloning of the encoding cDNA, reveals that it is a novel 2453 aa protein in which the nuclear receptor interaction domain and major repression domains are located in the extreme C- and N-terminal regions, respectively. Our data indicate that retinoic acid and thyroid hormone ligand-independent repression requires the recruitment of the co-repressor, N-CoR, that itself harbors the repressor domain acting via high affinity specific interactions with the thyroid hormone and retinoic acid receptors. This recruitment-based repression is reminiscent of the mechanisms of repression by HLH heterodimers via interactions with "groucho" in Drosophila and by MATα2/MCM1 via recruitment of the SSN6/TUP1 repressor in yeast. Indeed, these data, in concert with the identification of activation domains implies that both the repression and activation functions of nuclear receptors requires the recruitment of specific co-repressor and co-activator proteins, that interact with distinct regions within the ligand-binding domain. Both the DNA site and the heterodimer protein modulate the recruitment of the critical co-regulatory molecules.

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APPENDIX

Figure 1: A 270 kDa factor interacts with T3 receptor heterodimers on DNA.
Panel A: Cotransfection assays in CV1 cells utilized CMV transcription units expressing GAL4/T3Rβ1 (aa 165-462) fusion protein and RSV-T3Rβ1. As reporters the T4T-Luciferase transcription unit, containing two copies of a synthetic DR+4 element linked to the RARβ2 TATA and initiator sequences and a reporter unit that places the luciferase gene under control of GAL4-binding sites (17mer) upstream of the thymidine kinase reporter were used for transfections. Repression is observed only in the absence of ligand. Panel B: Identification of a 270 kDa protein that interacts with T3 receptor/retinoic X receptor heterodimers on a DR+4 site. Purified T3Rβ1 and RXRα were bound to the double-stranded, biotinylated oligonucleotide encompassing a DR+4 site. Protein, DNA complexes were captured on strepavidin agarose and incubated with CV1 whole cell extract in the presence or absence of 10^{-7}M Triac (3,3',5-trijodothyroacetic acid). The material was extensively washed, and proteins interacting with the T3R/RXR heterodimers in this DNA complex were recovered from the stepavidin agarose by boiling in SDS sample buffer, resolved by polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Proteins recovered that were capable of direct interaction with T3R were detected using unliganded ^{32}P-GST-T3Rβ1 C-terminus probe. With this assay, the strongest signal at 52-54 kDa represents binding of the probe to RXR and T3 receptors retained by the streptavidin-agarose matrix. The only detectable protein bound in a ligand-independent fashion was a 270 kDa protein (arrow). Panel C: The novel protein interacts with thyroid hormone and retinoic acid receptor in a hormone-independent manner. Fusion proteins of LexA (aa 1-202) and nuclear receptor C-termini were expressed in yeast and tested utilizing the two hybrid system. The novel protein was expressed as a fusion protein with the B42 activation domain provided by the "prey-vector" pJG4-5. The following baits were tested and the results of the interaction studies are shown above: $LexA_{(1-202)}$-$T3R\alpha1_{(122-410)}$, $LexA_{(1-202)}$-$RAR\alpha_{(143-462)}$, $LexA_{(1-202)}$-$RXR\gamma_{(134-463)}$, $LexA_{(1-202)}$-$VDR_{(88-427)}$, $LexA_{(1-202)}$-$ER_{(251-595)}$, and $LexA_{(1-202)}$-$GR_{(465-795)}$. Yeast (S. cerevisiae, EGY48) containing the reporter plasmid (pSH18-34), the prey (novel protein fused to B42), and the different bait constructs were grown over night. Assay cultures with galactose as carbon source were started with those overnight cultures and the β-Galactosidase activity was determined. The added hormones were 10^{-7}M Triac (first panel) and 10^{-7}M 9-cis retinoic acid (second panel).
Methods : **A.** Cotransfection studies were performed in 60 mm dishes using CMV/GAL4/$T3R\beta1_{(165-462)}$ fusion protein, RSV/T3Rβ1, T4T-Luciferase, and GAL4-tk-Luciferase as transcription factor and reporter plasmids, as previously described. The results are an average of triplicate determinations, differing by less than 10%. **B.** Oligonucleotides for the DR+4 site (sense strand: 5' ^{Bio}TTC TGG AGG TGA CAG GAG GAC AGC CC 3'; antisense strand: 5' ^{Bio}AAG GGC TGC TGT CCT CCT GTC ACC TCC AG 3') were synthesized to contain biotin residues at the 5' end using biotin-phosphoramidite; oligonucleotides were purified by acrylamide gel electrophoresis, annealed, and repurified. GST fusion proteins of hT3Rβ and hRXRα were expressed in E. coli and the GST portion was removed by thrombin cleavage. Binding was performed using 1 μg double stranded oligonucleotide and 100 ηg of hT3Rβ and hRXRα in binding buffer (8mM Tris-phosphate (pH 7.4), 1.2M KCl, 8% Glycerol, 4mM DTT, and 0.5% CHAPS (Boehringer Mannheim)). Receptor-DNA complexes were purified using streptavidin agarose (25 μl), washed three times (20mM Hepes (pH 7.7), 50mM KCl, 20% Glycerol, 0.05 % NP40). Receptors were then incubated in binding buffer, with or without 10^{-7}M Triac, at 21°C for 30 minutes. CV1 whole cell extracts (1.5 μg per lane), prepared as previously described, were added and incubated for 60 minutes at 4°C, then washed three times with binding buffer. Proteins were rescued from streptavidin agarose by boiling one minute in SDS sample buffer, resolved by electrophoresis on SDS, polyacrylamide gels, and transferred to

nitrocellulose membranes. The T3Rβ probe was prepared by inserting a fragment encoding an aa 13-456 of hT3Rβ into the vector pGEX-2TK to introduce a phosphorylation site for cardine kinase. The GST-T3Rβ fusion protein was phosphorylated using γ[^{32}P]ATP and used to detect proteins to nitrocellulose filters as previously described (reference). **C.** The yeast (S. cerevisiae) strain EGY48 was transformed with "bait-vector" (pEG202-T3R$\alpha_{122-410}$) (His3-plasmid) and the lacZ reporter plasmid pSH18-34 (Ura3-plasmid). A large scale high efficiency transformation was used to then introduce the HeLa oligo dT primed fusion library in pJG4-5 ("prey-vector") into the yeast clone. Approximately 1 x 10^6 yeast colonies were assembled on 140 150mm plates. The colonies were pooled using scraping buffer (65% glycerol, 10mM Tris-HCl pH 8.0, and 0.5mM EDTA),. Aliquots of this yeast stock were frozen at -70^{o}C. For the selection the yeast cells were grown for 4 hours in -Ura-His-Trp medium with 2% galactose as carbon source. 3.5 x 10^7 yeast cells were than plated out on -Ura-His-Trp-Leu medium containing 2% galactose as carbonsource. After 5 days approximately 4000 yeast colonies were obtained. In order to distinguish between false positives and real interactors 400 yeast colonies were replica plated on -Ura-His-Trp galactose medium containing 0.1% Xgal. Colonies that showed a dark blue color after two days on these plates were assayed for β-Galactosidase activity after being grown in liquid culture (-Ura-His-Trp medium) with both, glucose and galactose as carbonsource as described. Yeast clones containing reporter, the different baits (LexA fusions with T3R$\alpha_{(122-410)}$-, RAR$\alpha_{(143-462)}$-, RXR$\gamma_{(134-463)}$-, VDR$_{(88-427)}$-, ER$_{(251-595)}$-, and GR$_{(465-795)}$-C-termini), and the prey were grown in 5ml over night cultures in -Ura-His-Trp medium with glucose as carbon source. Assay cultures (-Ura-His-Trp medium) with galactose as carbonsource were grown to OD_{600} 0.4-0.8 and β-Galactosidase activity was determined as described.

Figure 2: Biochemical characterization of N-CoR interactions and identity.
Panel A: Biochemical demonstration of p270/N-CoR, T3R or p270/N-CoR, RAR interactions. The ability of C-terminal 825 aa fragment of N-CoR (aa 1628-2453) or the holoprotein, prepared by in vitro transcription, translation in rabbit reticulocyte lysate TNT, were used to bind to bacterially-expressed Glutathione-S-transferase (GST) fusion proteins of full length thyroid hormone, retinoid acid and retinoic X receptors. Binding was assessed in the presence and absence of ligand (Triac (10^{-7}M), retinoic acid (10^{-7}M), and LG69 (10^{-7}M)). Binding of thyroid hormone receptor and retinoic acid receptor was comparable in five experiments of similar design; binding to RXR was minimal, or undetectable, in five independent experiments. In contrast to binding on DNA, ligand was less effective in preventing binding of p270/N-CoR to RAR: Triac (3,3',5-triiodothyroacetic acid) (A) but not T3 (3,3',5-triiodo-L-thyronine) resulted in significant loss of N-CoR binding to GST-T3R fusion proteins. Panel B: PolyA-selected RNA from a series of murine tissues have been subjected to fractionation on denaturing gels, transferred to membranes, and hybridized against a N-CoR-specific DNA-probe; a single mRNA species migrated ~9.2 kb. RNase protection assays were conducted with a large number of tissues and cell lines, a few of which are included in penal A. N-CoR transcripts were detected in every cell line and tissue analyzed, but some wide variations in signal were noted. Panel C: N-CoR is a nuclear protein and is identical to the p270 that binds to TR *in vitro*. The cDNA encoding N-CoR protein, containing an in-frame C-terminal 14 amino acid extension, ([EYKEEEK]$_2$), corresponding to the sequence of the "FLAG" epitope was generated and expressed under the control of the CMV promoter in COS cells. Use of the anti-FLAG monoclonal antibodies (Kodak Biosystems) permitted detection of the N-CoR protein in the nucleus, but not cytoplasm of transfected cells; untagged protein was not detected. Western blot analysis confirmed specificity and nuclear localization of N-CoR. Immunoprecipitation of the thyroid hormone receptor with a T3R antiserum result in co-immunopreceptation of N-CoR, detected using anti-Flag antibodies. To confirm that the 270 kDa protein biochemically detected on ABCD or GST pull down assays with thyroid hormone or retinoic acid receptors corresponded to the cloned 270 kDa protein, the

migration of the protein in 4 cell extracts bound by T3R/RXR heterodimers on a DR+4 site detected using ^{32}P-labeled T3-receptor or retinoic acid receptor C-terminus was compared with the protein bound to GST-T3R or GST-RAR fusion proteins (western blot), detected using ^{32}p-RAR c-terminus probe or a polyclonal antiserum against N-CoR. The apparent co-migration of the two proteins, indicated that N-CoR represented the p270 factor detected biochemically. P270 was purified using bacterially-expressed and detected with either 35P GST, RAR (2) or anti N-CoR antisense (R).

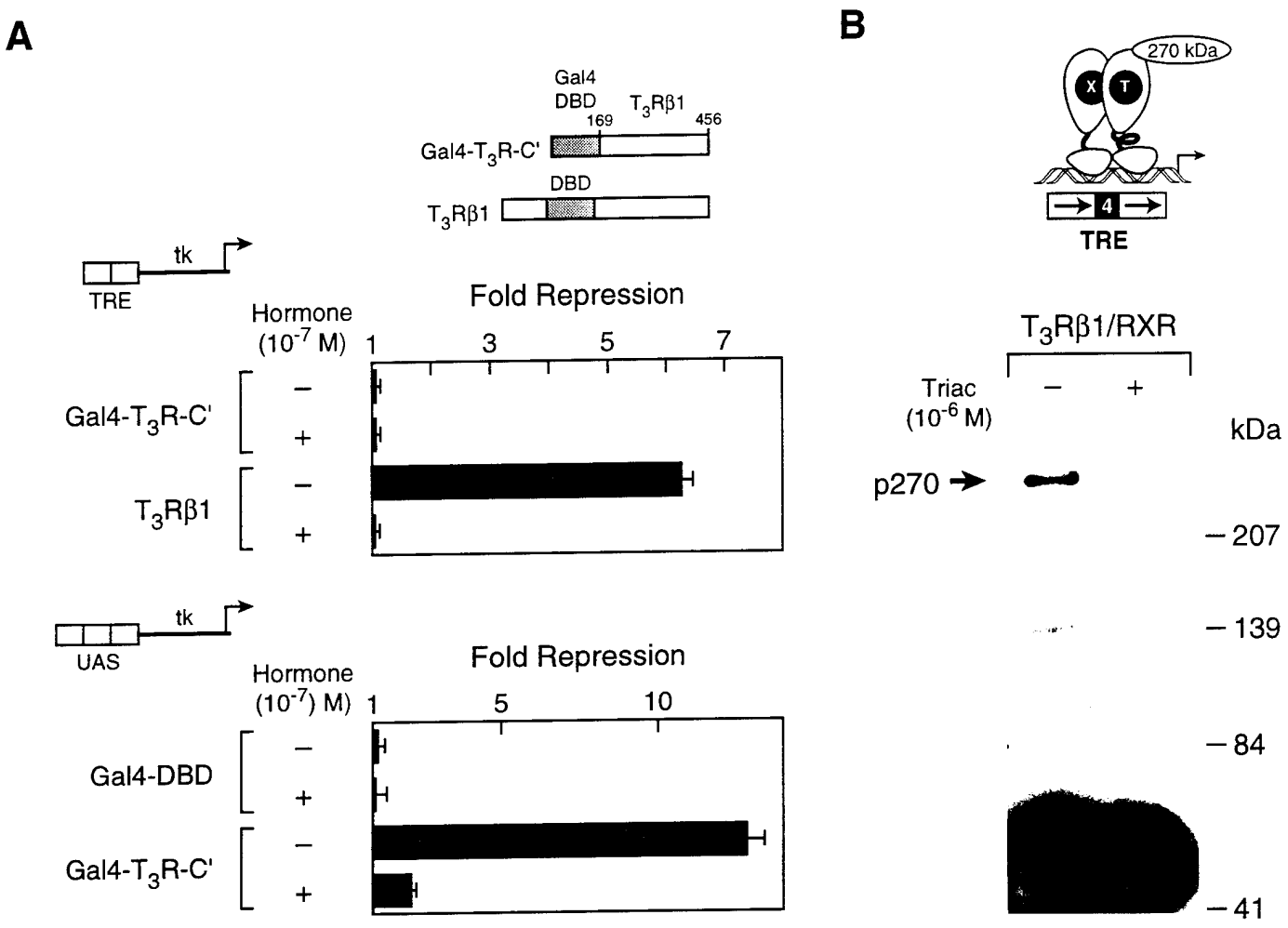
Methods : **A.** GST fusion proteins of hRARα, hT3Rβ, and hRXRα were prepared and purified on glutathione agarose as previously described and ^{35}S-labeled full length N-CoR or N-CoR 100 (C-terminal 825 aa) was prepared by *in vitro* transcription/translation (TNT). These ^{35}S-Methionine labelled proteins were incubated for 20 minutes at 37°C with 2-3μg of GST fusion protein bound to 25μl of glutathione agarose beads in a total volume of 100μl of 20mM Hepes (pH7.9), 100mM NaCl, 1mM EDTA, 4mM MgCl2, 1mM DTT 0.02% Nonidet P40, 20% glycerol, and 0.5% nonfat dry milk, washed, eluted, and separated by SDS, polyacrylamide gel electrophoresis. Similar results were obtained in three independent experiments. **B.** PolyA RNA from adult murine tissues (2 μg per lane) size fractionated using denaturing gelelectrophoresis and transferred to a nylon membrane were hybridized with a ^{32}P-labeled DNA probe corresponding to aa 791-1147 of N-CoR. The RNase protected fragment, a 389 nucleotide piece corresponding to aa 1715-1844 was cloned in 3' to 5' direction (reading frame of N-CoR) into pSP65 using the restriction endonucleases PstI/SmaI (Vector) and PstI/PvuII (N-CoR fragment). A 420 nucleotide long, ^{32}P-labeled SP6-RNA transcript was generated. The control was provided using α, β actin probe, as previously described (reference). **C.** Two oligonucleotides corresponding to two copies of the FLAG sequence , including a BamHI site (5') and a NotI site (3') for cloning, were synthesized (sense: 5' GCG GGA TCC GAC TAC AAG GAC GAC GAT GAC AAG GAC TAC AAG GAC GAC GAT GAC AAG TGA GCG GCC GCG GGC CCA GAG 3'; antisense: 5' CTC TGG GCC CGC GGC CGC GCA CTT GTC ATC GTC GTC CTT GTA GTC CTT GTC ATC GTC GTC CTT GTA GTC GGA TCC CGC 3'), phosphorylated annealed, digested with BamHI and NotI, and fused in frame at the C-terminus of N-CoR. The "flagged" C-terminus was then used to build a cDNA encoding for full length N-CoR in a four step construction. This cDNA was placed under the control of a CMV transcription unit. COS cells were transfected and utilized for immunohistochemical analysis using the IBI FLAG monoclonal antibody and fluorescent secondary antibody. For the western blot analysis of p270 (N-CoR) the nitrocellulose membrane of SDS, PAGE separated GST-RAR bound CV1 extract was incubated with a guinea pig polyclonal antiserum raised against recombinant p270 at a 1:500 dilution; detection was performed using Vectostain ABC Kit (Vector Laboratories) ELC western blotting system (Amersham). The p270 bound to T3R/RXR heterodimers detected by ^{32}P-labeled T3R probe, as described in the legend to Fig. 1.

Figure 3: Mapping of the N-CoR interaction domain to a conserved region in the hinge of T3R and RAR.

Panel A: Mapping of the interaction domain of N-CoR. Using PCR techniques a series of 5 different C-terminal an 1 N-terminal deletions of the N-CoR C-terminus (aa 2240-2453) were generated and cloned into pJG4-5 ("prey-vector") The interaction of each of these constructs were studied using LexA-T3Rα1$_{(122\text{-}410)}$ as a bait. Assays for β–Galactosidase in liquid cultures from yeast (S. cerevisiae) containing this bait, reporter plasmid pSH18-34, and the different prey constructs were performed as described above. The smallest fragment that was still able to interact with thyroid hormone receptor was a 60 amino acid (aa 2240-2300) stretch in the C-terminus of the protein. Panel B: Summary of results of N-CoR binding to a series of T3-receptor N'- and

C'-terminal deletion proteins, determined using either GST-N-CoR C-terminus, GST-T3Rα1, or GST-T3Rβ1 fusion proteins and ^{35}S-methionine labeled T3Rβ or N-CoR 100 (C-terminus) proteins in a GST co-immunoprecipitations assay. The T3Rβ past aa 260 failed to bind N-CoR; no specific sequences are required C-terminal of residue 260. N-terminal deletions up to residue 203 retain full interaction, while deletions to aa 230 results in full loss of this activity. Panel C: GST pull down assays of TNT translated ^{35}S-methionine labeled T3Rβ1 variants. PCR techniques (in case of T3Rβ1$_{(1-456)}$, T3Rβ1$_{(1-421)}$, T3Rβ1$_{(1-381)}$, T3Rβ1$_{(1-335)}$, and T3Rβ1$_{(1-181)}$) and digestion with restriction endonucleases (in case of T3Rβ1$_{(\Delta 260-335)}$) were used to generate cDNA's encoding for five different C-terminal and internal variants of T3Rβ1. These cDNA's were utilized to generate ^{35}S-labelled proteins. GST pull down assays were performed with a fusion of GST and the C-terminus (aa 2057-2453) of p270 as described above. Bound (B) and input (I) of the different ^{35}S-labelled proteins are indicated. Panel D: GST pull down assays of TNT translated ^{35}S-methionine labelled p270 C-terminus. Using PCR techniques four different cDNA's encoding different GST-T3Rβ1 fusion proteins (GST-T3Rβ1$_{(165-456)}$, GST-T3Rβ1$_{(186-456)}$, GST-T3Rβ1$_{(203-456)}$, and GST-T3Rβ1$_{(230-456)}$) were generated. GST pull down assays were performed with ^{35}S-methionine labelled p270 C-terminus (aa 1629-2453) as described above.

Methods : **C+D.** Thyroid hormone receptor mutants were generated using appropriate synthetic oligonucleotides to synthesize PCR fragments or naturally occurring restriction sites in the hT3Rβ cDNA, which were subjected to DNA sequencing and used to generate cDNA's encoding GST-fusionproteins or directly cloned into pBKS inserts for TNT reactions. In each case binding was assessed by GST coprecipitations assays.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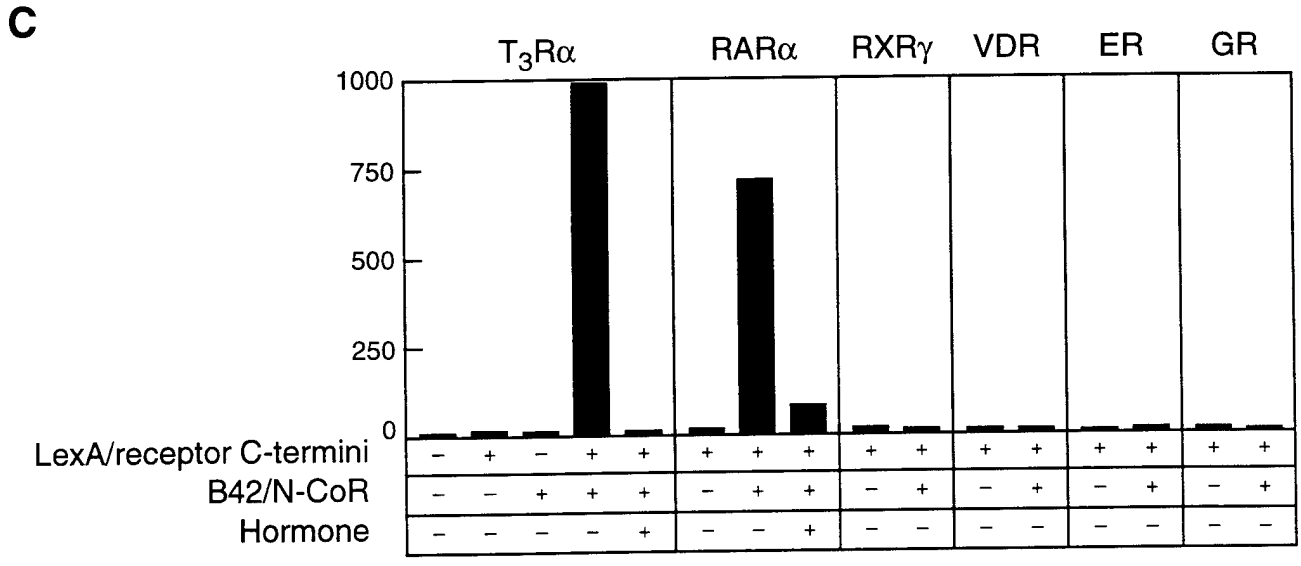
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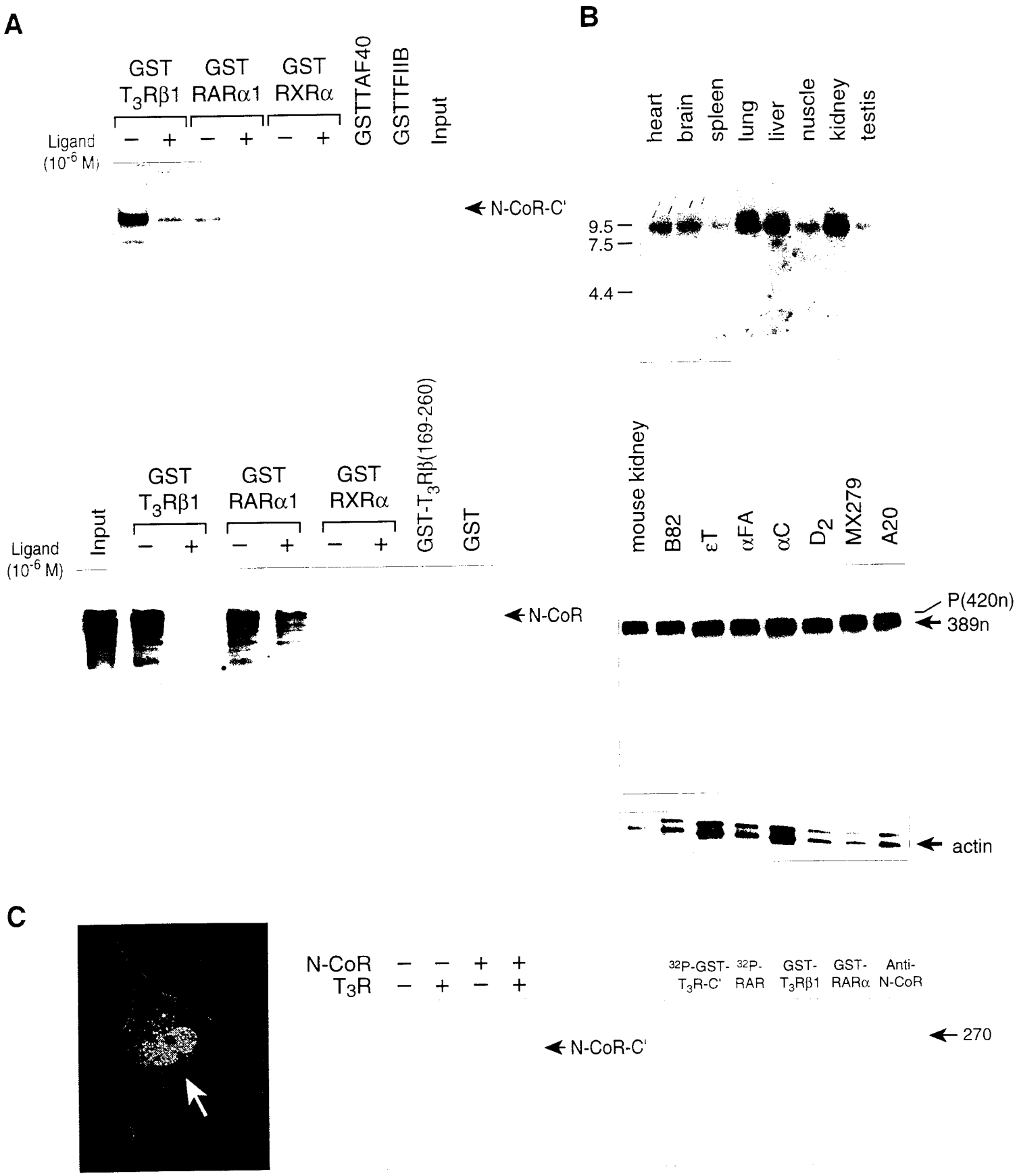
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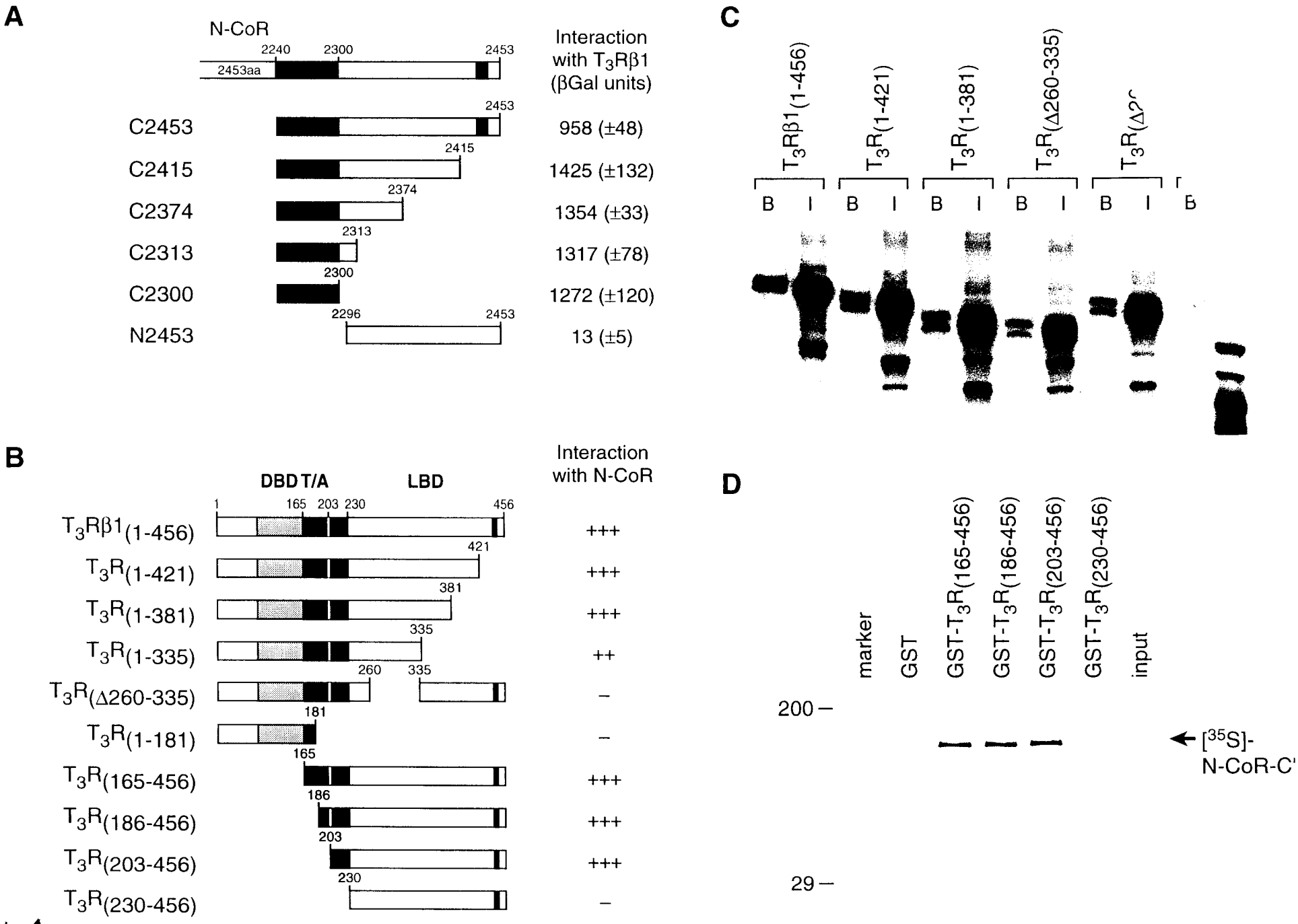
FIGURE 3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B
DBD
T/A
LBD
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+++
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T3R(1-335)
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